Comprehensive diagnosis of Rett's syndrome relying on genetic, epigenetic and expression evidence of deficiency of the methyl-CpG-binding protein 2 gene: study of a cohort of Israeli patients

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ELECTRONIC LETTER

Comprehensive diagnosis of Rett's syndrome relying on genetic, epigenetic and expression evidence of deficiency of the methyl-CpG-binding protein 2 gene: study of a cohort of Israeli patients

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Background: Despite advances in the characterisation of mutations in the *MECP2*-coding region, a small proportion of classic RTT cases remain without recognisable mutations.
Objective and methods: To identify previously unknown mutations, a quantitative assay was established, providing estimates of MECP2_e1 and MECP2_e2 expression levels in peripheral blood. A systematic analysis of an Israeli cohort of 82 patients with classic and atypical RTT is presented, including sequence analysis of the *MECP2*-coding region, MLPA, XCI and quantitative expression assays.
Results and conclusion: A novel mis-sense mutation at ca 453C→T (pD151E), resulting in a change of a conserved residue at the methyl-binding domain, and a rare GT deletion of intron 1 donor splice site are reported. It is shown that various *MECP2* mutations had distinct effects on MECP2 expression levels in peripheral blood. The most significant ($p<0.001$) reduction in the expression of both MECP2 isoforms was related to the presence of the intron 1 donor splice-site mutation. Using quantitative expression assays, it was shown that several patients with classic and atypical RTT with no mutation findings had significantly lower MECP2 expression levels. Further research on these patients may disclose still elusive non-coding regulatory MECP2 mutations.

Key points

- This study introduces a novel assay providing quantitative estimates of expression levels of both MECP2_e1 and MECP_e2 isoforms in peripheral blood that could support the diagnosis of patients with Rett syndrome (RTT) without recognisable MECP2 mutations.
- We implemented these quantitative expression assays in a systematic analysis of an Israeli cohort of RTT patients together with analyses of *MECP2* coding sequence, MLPA and XCI.
- We show that quantitative expression assays distinguish between various *MECP2* mutations, with significantly reduced peripheral MECP2 expression in cases with the intron 1 donor splice-site mutation and normal expression in cases with mis-sense mutations and small in-frame deletions. In addition, we show several classical and atypical patients with no previous mutation findings that have lower peripheral MECP2 expression levels, which suggest the possibility of yet unidentified *MECP2* mutations.

Rett syndrome (RTT; OMIM 312750) is a neurodevelopmental disorder characterised by cognitive impairment, communication dysfunction, microcephaly, growth failure and stereotypic hand movements. RTT primarily affects females and is sporadic in most cases, with a worldwide incidence of 1/10 000–15 000 female births.[1] In most cases, symptoms of RTT become apparent during the initial years of life, when patients fail to achieve or regress from the expected developmental milestones and become engaged in characteristic hand-wringing motions. Most of the patients in addition have breathing abnormalities, about a half of them develop seizures and a third develop scoliosis. By age 4–7 years, most patients develop gross cognitive and motor impairments, leading to profound lifelong hypoactivity.[2 3] The diagnosis of RTT is essentially clinical and is based on fulfilment of consensus criteria for both the classic and atypical forms; atypical RTT includes forme fruste, preserved speech variant (PSV), late regression variant, and congenital and early seizure onset variants.[4] RTT has been associated with mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene,[5] and additional clinical phenotypes have been included on genetic basis, among them Angelman-like phenotype and familial X-linked metal retardation, and autism in males (reviewed by Naidu *et al*[6]).

From the genetic standpoint, RTT is an X-linked dominant disorder caused by defects in the *MECP2* gene on chromosome Xq28. *MECP2* mutations were detected in >85% of patients with classic RTT, most of which (about 70%) involve C→T transitions at specific CpG hot spots in exons 3 and 4; an additional 10% involve small deletions at the 3′ end of the gene.[7] After the finding of the novel MECP2_e1 transcription isoform,[8 9] mutation analysis of *MECP2* has been extended to include exon 1 that was previously considered untranslated. The use of novel molecular technologies, such as denaturing high-performance liquid chromatography, multiple ligation-dependent probe amplification (MLPA) and genomic-based quantitative polymerase chain reaction (PCR),[10–12] has increased the overall detection rate of *MECP2* mutations in patients with classic RTT to nearly 90%. An additional 15% of

Abbreviations: MECP2, methyl-CpG-binding protein 2; MLPA, multiple ligation-dependent probe amplification; PCR, polymerase chain reaction; PSV, preserved speech variant; QtPCR, quantitative PCR reaction; RTT, Rett's syndrome; UTR, untranslated region; XCI, X chromosome inactivation

atypical RTT cases, particularly early seizure onset variants, have been resolved by the recent findings of mutations in the X-linked cyclin-dependent kinase-like 5 gene.[13 14] However, despite these advances in the molecular diagnosis of RTT, the remaining 10% of patients with classic RTT and >50% of those with atypical RTT continue to pose a diagnostic dilemma. As *MECP2* is located on the X chromosome, another modulator of the RTT phenotype is the X chromosome inactivation (XCI) pattern. Preferential activation of the normal allele is one of the possible explanations of asymptomatic female carriers of RTT-causing mutations or those having minor learning disabilities.[15]

The conventional means of obtaining molecular evidence of RTT has been based, thus far, on sequencing of the *MECP2*-coding region and exon–intron junctions. At the same time, mutations in the regulatory elements of *MECP2*, which ultimately affect MECP2 expression levels, have been omitted from this analysis. We considered an additional approach to the molecular diagnosis of RTT on the basis of the analysis of MECP2 expression levels in peripheral blood of patients. We developed a rapid and direct assay providing quantitative estimates of expression levels of both MECP2_e1 and MECP2_e2 transcription isoforms in peripheral blood, using real-time PCR and fluorescent-labelled isoform-specific TaqMan probes. This approach could be potentially diagnostic in patients in whom no *MECP2*-coding mutations have been previously detected. Accordingly, we preferentially implemented MECP2 expression assays in a group of Israeli patients and some patients referred by our US and Canadian colleagues, in whom no *MECP2* mutations had been detected. In addition, MECP2 expression assays were also carried out in some representative cases with *MECP2* mis-sense and nonsense mutations, C-terminal deletions and large rearrangements in the *MECP2* gene region.

METHODS

Patients and controls

The Israeli RTT cohort included 82 patients of whom 52 were diagnosed with classic and 17 with atypical RTT, including 4 with PSV, 4 with congenital variants, 6 with early seizure onset variants, 2 with forme fruste variants and 1 male variant, using clinical criteria established by Hagberg *et al*.[4] The cohort also included four females with Angelman-like features and nine patients with diagnoses reminiscent of RTT, including seven females with autism spectrum disorder and two males with congenital severe encephalopathy. All the patients were classified by the same paediatric neurologist at the Neuropediatric Clinic at the Sheba Medical Center, Tel Hashomer, Israel, where they continue to attend for clinical follow-up. The age of patients was in the range 2–40 years. Molecular diagnosis of RTT was carried out as a part of an ongoing study approved by the institutional review board or as an officially authorised diagnosis provided by health insurance. Patients included in the expression studies were recruited after informed consent of the parents approved by the institutional review board. This group included patients with classic (C1–C5) and atypical (A1–A7) RTT. Patients C1–C5 and A1, A3 and A4 were recruited from the Israeli cohort, patient A5 was referred from the Hospital for Sick Children, Toronto, Ontario, Canada, and patients A2, A6 and A7 were referred from the Rett Syndrome Research Foundation, Cincinnati, Ohio, USA. As regards the diagnoses of patients with atypical RTT, A1 and A2 had congenital variants, and A3 and A4 had early seizure onset variants, but A5, A6 and A7 had no specific diagnoses other than atypical RTT. In addition, a sample from an atypical patient with partial deletion of exon 4 (del exon 4a) was provided by Dr Jane Hickey via the Rett Syndrome Research Foundation. Expression studies also included 12 samples from healthy female volunteers (F1–F12) aged 17–40 years.

DNA and RNA extractions

Peripheral blood samples collected in tubes containing EDTA anticoagulant were used for the extraction of genomic DNA by the Puregene kit and total RNA by the Versagene kit (both by Gentra, Minneapolis, MN, USA). Additional blood samples were transferred from Canada and the US in Paxgene tubes (PreAnalytix, Hombrechtikan, Switzerland) and RNA was extracted by the same method. The yield and purity of the DNA and RNA extractions were determined according to the optical density ratio 260/280 nm using a spectrophotometer (GeneQuant, Cambridge, UK). RNA samples were subjected to DNAse I treatment included in the Versagene kit; 10–20 units of protector RNAse inhibitor (Roche Applied Science, Penzburg, Germany) were added and the samples were stored at −20℃.

MECP2 mutation analysis

PCR fragments spanning the *MECP2* promoter region (1.2 kb upstream of exon 1) and the entire coding region (exons 1–4) were generated using seven primer pairs (sequences available on request). PCR fragments were purified on silica gel spin columns (Bioneer, Daejon, Korea) and analysed on an ABI Prism 3100 AVANT Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The presence of mutations was confirmed by replicate sequencing of independently generated PCR products.

Multiplex ligation-dependent probe amplification

Large deletions spanning *MECP2* exons 1–4 were detected using the MLPA-P015 probe (MRC, Amsterdam, The Netherlands) according to the manufacturer's protocol. MLPA products were separated and identified using the ABI Prism 3100 Genetic Analyzer using Genescan ROX-500 standards and Genescan software (both by Applied Biosystems). Reproducible deviation of >30% of relative peak area as compared with the normal control sample derived from two independent experiments was considered conclusive.

X chromosome inactivation

The XCI pattern in peripheral blood was analysed using the human androgen receptor assay.[16] Briefly, genomic DNA was digested with the methylation-sensitive enzyme *Hpa*II for preferential cleavage of the unmethylated-active alleles. Digested and undigested DNA samples were subjected to PCR of the human androgen receptor gene, including the highly polymorphic trinucleotide repeat region. PCR products were separated using the ABI Prism 3100 Genetic Analyzer, calculating the peak areas of the undigested and digested alleles using Genescan ROX-500 standards and Genescan software. Estimation of XCI included correction for the large digested allele peak area considering the ratio between peak areas of the small and the large undigested alleles. Skewed XCI was considered to be significant for ratios ⩾75%, whereas reported values were derived from two independent and reproducible experiments (<10% difference). The parental origin of the preferentially activated chromosome was further determined by comparing the profiles of the patient's and the parental samples.

cDNA synthesis

Double-stranded cDNA was generated from 500 ng of total RNA in the presence of $p[dN]_6$ random primer hexamers (Roche Applied Science), using an Omniscript RT kit (Qiagen Hilden, Germany) according to the manufacturer's protocol. Reactions were carried out in a total volume of 40 μl.

TaqMan reactions

Relative expression levels of MECP2_e1 and MECP2_e2 transcription isoforms were determined using primers designed by Primer Express software, TaqMan probes and PCR kit (all from Applied Biosystems). The MECP2_e1 assay, designed by us, included forward primer from exon 1 (5′-CGG AGG AGG AGG AGG A) and reverse primer from exon 3 (5′-GGA GGT CCT GGT CTT CTG ACT T), producing a 63-bp amplicon, and the TaqMan probe for the exon 1–3 junction (gene accession BX538060). The MECP2_e2 commercial assay included the TaqMan probe for the exon 2–3 junction. Both MECP2 probes contained 5′-6-carboxyfluorescein fluorophore reporter and 3′-non-fluorescent quencher. The commercial RNAseP kit consisting of RNAseP-specific primers and VIC-labelled TaqMan probe was used as an internal reference, enabling multiplexing of MECP2 and RNAseP assays. Separate pre-runs of varying primer concentrations were carried out to obtain the highest intensity and specificity of the fluorescent reporter signal. The results were validated in separate reactions including the commercial 6-carboxyfluorescein-labelled ornithine decarboxylase 1 probe and primers as another reference. Quantitative PCR reactions (QtPCRs) were carried out in a volume of 20 µl in 96-well optical plates using a common Mastermix including 2× TaqMan Universal PCR Mastermix, 900 nM MECP2_e1 or MECP2_e2 primers, 250 nM MECP2_e1 or MECP2_e2 probe and 20× RNAseP kit, and high-performance liquid chromatography-pure water. Aliquots of 2 µl of each cDNA species were dispensed in three wells for triplicate reactions. Reactions were carried out on ABI Prism 7000 (Applied Biosystems) under uniform conditions, including a pre-run at 50℃ for 2 min and 95℃ for 10 min, and 40 cycles at 95℃ for 15 s and at 60℃ for 1 min. Each reaction plate included triplicate samples of a normal control pool constructed from 4–5 cDNAs generated from normal females (fig 1). Each plate also included reactions with no-template control and RNA template (RT) for monitoring of the background signal and DNA contamination, respectively.

Quantitative analysis

Data were evaluated using the ABI Prism 7000, comparing the levels of MECP2_e1 and MECP2_e2 transcripts with those of the RNAseP internal reference gene. In most cases, the threshold of QtPCRs was automatically set at 10 standard deviations (SD) above the mean baseline emission, representing the background signal, and the specific reaction cycle threshold (Ct) number was determined relative to this threshold. Only samples with at least three QtPCR results (triplicates) were included in the data analysis. The comparative (ddCt) method was used to calculate the relative transcript number,[17] previously verifying that QtPCR efficiencies of the target and reference genes were almost equal. Efficiencies of MECP2_e1, MECP2_e2 and RNAseP reactions were determined from standard curves generated by serial twofold dilutions of the cDNA sample and estimation of the cycle threshold at each dilution (fig 2). Reaction efficiencies, calculated as E (efficiency) $= 10^{-1/\text{slope}}$, yielded values of 1.84, 1.86 and 1.89 for MECP2_e1, MECP2_e2 and RNAseP, respectively, which could be considered approximately equal (standard error <0.02). Relative transcript number in the patient samples was determined according to the ddCt method comparing the patient's sample and the pooled sample of normal female controls using the following equations:

$$dCt = Ct_{MECP2} - Ct_{RNAseP} \text{ and } ddCt = dCt_{patient} - dCt_{control\ pool}$$

and on inclusion of a correction for mean PCR efficiency,

$$\text{MECP2\_e1 transcript number} = 1.86^{-(ddCt)},$$

$$\text{MECP2\_e2 transcript number} = 1.84^{-(ddCt)}$$

Statistical methods

For the purpose of statistical analyses of MECP2_e1 and MECP2_e2 expression data, patients were grouped into four groups according to the MECP2 mutation type (I, splice-site mutations; II, large deletions; III, nonsense and frame-shift mutations; and IV, mis-sense and in-frame mutations). The individual mean MECP2_e1 and MECP2_e2 levels in the patient groups and those in the normal control group were compared using analysis of variance with Bonferroni correction for multiple comparisons. Correlation analysis between the levels of both MECP2 isoforms was carried out using the Spearman correlation procedure. Multinomial (polytomous) regression was applied to predict the relatedness of patients with no known *MECP2* mutation to mutation groups I–IV, while combining groups I and II. All data were analysed using SAS V.9.1.3 for Unix via procedures MIXED and LOGISTIC.

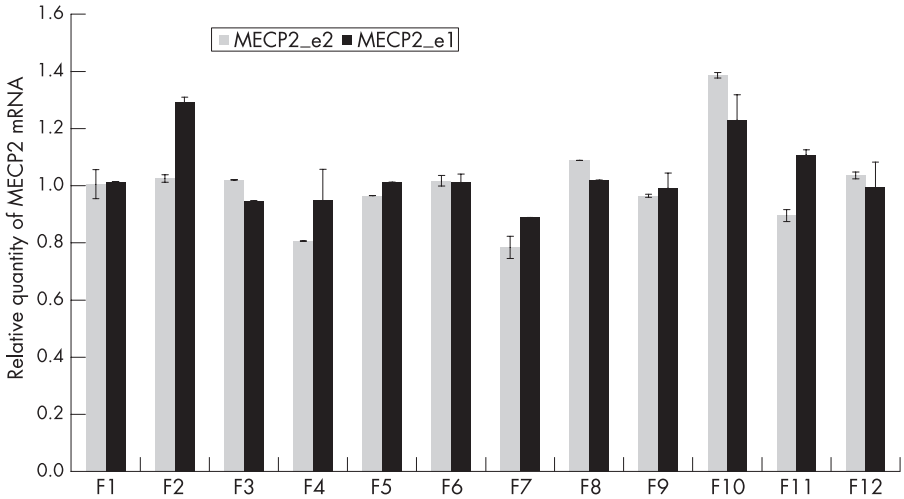

Figure 1 Quantitative analysis of peripheral methyl-CpG-binding protein 2 (MECP2)_e1 and MECP_e2 expression levels in healthy female controls. Expression levels of both MECP2 isoforms in normal female control samples (F1–F12) were determined according to the ddCt method using RNAseP as a reference gene. MECP2_e1 (grey) and MECP2_e2 (black) expression levels were normalised to the mean dCt value.

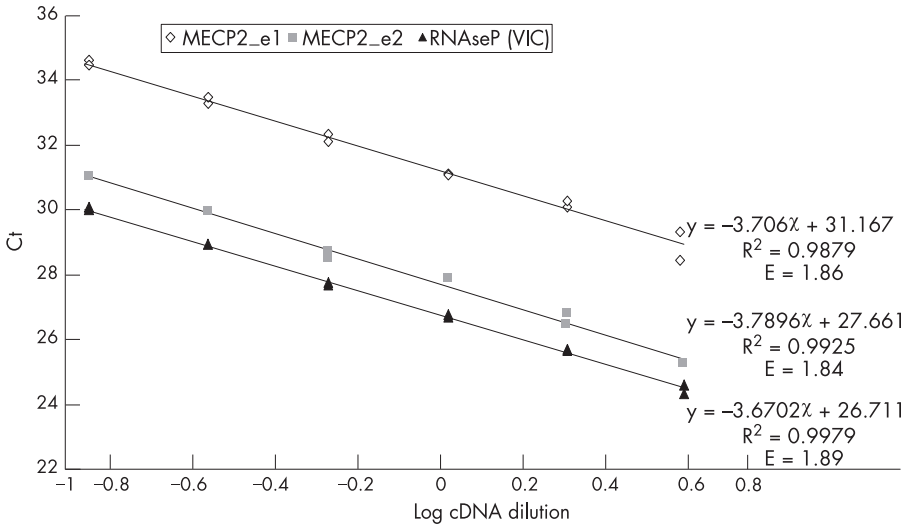

Figure 2 Validation of amplification efficiencies of the target methyl-CpG-binding protein 2 (*MECP2*) gene and reference RNAseP gene transcripts. Standard curves plotting the log of cDNA dilutions versus cycle threshold (Ct) were generated in duplicates by serial (factor 2) dilutions of the cDNA sample. Quantitative polymerase chain reaction analyses were carried out in the presence of TaqMan probes specific to the MECP2_e1 exon 1–3 junction (white), MECP2_e2 exon 2–3 junction (grey) and exonic RNAseP (black). Efficiencies of each assay were calculated from the curve slopes ($E = 10^{-1/slope}$).

RESULTS

Analyses of *MECP2* mutations, MLPA and XCI

MECP2 mutation analysis of the Israeli cohort (n = 82) yielded 54 patients with *MECP2* sequence variations, including 42 with classic RTT, 4 with PSV, 2 with congenital variants, 1 with forme fruste variant and 5 with atypical RTT. Our mutation detection rate is 80% (42/52) as regards patients with classic RTT. Table 1 summarises the clinical and mutation data of patients, including XCI regarding preferential activation of the maternal or paternal X chromosome. Apart from the known *MECP2*-coding mutations and various microdeletions at the 3′-coding end, we identified a novel mis-sense mutation at c453C→G (p D151E) in one patient with PSV, located at the MECP2 methyl-binding domain. In two patients with classic RTT, we detected a rare GT deletion in an intron 1 donor splice site. In one patient with classic RTT, we detected changes in both *MECP2* alleles, including a 1.1-kb deletion in the intron 3–exon 4 region and a novel maternally inherited sequence variation at c824T→C (p V275A). Using MLPA, we detected a large (⩾4.2 kb) deletion spanning parts of exon 4 and the 3′ untranslated region (UTR) in one congenital variant. We also detected four maternally inherited sequence variations (table 1, lower panel), among which c378-19delT, c602C→T (p A201V) and c1451G→C (p R484T) have been reported in the International Rett Syndrome Association database as polymorphisms, but c753C→T (p P251P) is a novel mutation. Among the patients with *MECP2* mutations, we detected significantly skewed (⩾75%) XCI in 11 of 48 (23%) informative cases, including three patients with preferential activation of the paternal X and eight patients with preferential activation of the maternal X chromosome. Ultimately, ten patients with classic RTT remained with no indication of *MECP2* mutation, either by sequence analysis or by MLPA. Five of these patients were further included in quantitative analyses of MECP2 expression in peripheral blood (C1–C5), together with seven patients with atypical RTT with no mutation findings (A1–A7).

Development of quantitative assay for the analysis of MECP2 expression levels

We developed an additional strategy for the detection of MECP2 deficiency, introducing quantitative analysis of MECP2 expression levels in peripheral blood, using TaqMan probes for the target genes, *MECP2_e1* and *MECP2_e2*, and *RNaseP* as a reference gene. QtPCR efficiencies of the target and reference genes were estimated from the cycle threshold values obtained from serial dilutions of a cDNA sample. Figure 2 shows that all QtPCR efficiencies were similar and within the range 92–95% of maximal efficiency. MECP2_e2 was detected at a lower cycle threshold (difference of 4Ct), suggesting that the levels of MECP2_e2 are ~8 times as abundant in the peripheral blood, which is also consistent with other reports.[9] The normal range of MECP2_e1 and MECP2_e2 expression levels in peripheral blood was estimated by the analysis of a series of normal female control samples (F1–F12). Figure 1 shows that in most cases, MECP2_e1 and MECP2_e2 expression levels in the normal female controls were analogous and differed by factor of 1.5 (0.09) for MECP2_e1 and 1.7 (0.14) for MECP2_e2. Thus, in the following expression assays we included a pooled sample of four cDNAs from normal female controls.

Analysis of MECP2_e1 and MECP2_e2 expression levels in patients with known mutations

To validate the consistency of quantitative expression assays with the presence of known *MECP2* mutations, we examined the expression levels of MECP2_e1 and MECP2_e2 in the peripheral blood of 15 such patients. Figure 3A,B shows this analysis. Dotted lines indicate the normal range of MECP2 expression in normal female controls. Grouping the patients into four mutation groups, including splice-site mutations, large deletions, truncating (nonsense and frame-shift) and non-truncating (mis-sense and in-frame) mutations, we compared MECP2 expression levels between these groups and the group of normal controls. We found significant differences in MECP2 expression levels between various mutation groups ($F = 19.01$, $df = 4$, $p<0.001$ for MECP2_e1; and $F = 23.01$, $df = 4$, $p<0.001$ for MECP2_e2 by analysis of variance and Bonferroni correction). The group with the splice-site mutations had significantly lower levels of both MECP2 isoforms than the normal controls ($p<0.001$ for MECP2_e1 and $p = 0.003$ for MECP2_e2 by analysis of variance and Bonferroni correction). The large deletions were lower specifically for MECP2_e2 ($p = 0.069$ for MECP2_e1;

Table 1 Characterisation of the Israeli cohort with Rett syndrom by analyses of mutation analysis of the methyl-CpG-binding protein 2-coding region, multiple ligation-dependent probe amplification and X chromosome inactivation

Rett phenotype	Nucleotide position	Amino acid change	Functional domain	XCI pattern (preferentially active X)
1 Classic (donor 1a)	c 62+1delGT	Splicing alteration	Intron 1 splice donor	R*
1 Classic (donor 1b)	c 62+1delGT+378-19delT	Splicing alteration	Intron 1 splice donor	S (90% paternal X)*
1 Classic	c 141insA	p E55fs57X	Up MBD	S (80% maternal X)
2 Classic	c 316C→T	p R106W	Up MBD	2 S (90%* and 80% maternal X)
1 Classic	c 378-219_1164del1018+824T→C	Rearrangement and p V275A	TRD	R
2 PSV, 2 classic	c 397C→T	p R133C	MBD	4 R
1 Classic	c 401C→G	p S134C	MBD	R
1 PSV	c 453C→G	p D151E	MBD	NI
1 Classic	c 473C→T+378-19delT	p T158M	MBD	R*
7 Classic	c 473C→T	p T158M	MBD	6 R, 1 NI
5 Classic	c 502C→T	p R168X	Inter-domain	3 R, 1 S (90% maternal X), 1 NI
1 Classic	c 674CC→TG	p P225R	TRD	R
1 Classic	c 731insC	p Q244fs258X	TRD	R*
8 Classic	c 763C→T	p R255X	TRD-NLS	6 R*, 1 S (80% maternal X), 1 NI
1 Classic	c 775_995del221	p A259fs266X	TRD-NLS	R*
1 Classic	c 806delG	p G269fs288X	TRD-NLS	S (80% maternal X)
2 Classic	c 808C→T	p R270X	TRD-NLS	2 S (75% maternal X and 75% paternal X*)
1 Congenital	c 808delC	p R270fs288X	TRD-NLS	R
1 Classic	c 880C→T	p R294X	TRD	R
1 Classic	c 908_1201del294	p I303_T400del98	TRD	R*
1 Classic, 1 FF	c 916C→T	p R306C	TRD	2R
1 Classic	c 1080 _1161del82	p P360fs365X	C-terminus	R
1 Classic	c 1157_1197del41	p L386fs486X	C-terminus	R
1 Classic	c 1157_1327del171	p L386fs431X	C-terminus	R*
1 PSV	c 1157_1198del42	p L386-S401del15insP	C-terminus	R*
1 Congenital (del exon 4b)	c TRD_3′ UTR ⩾4.2 kb del	Large rearrangement	C-terminus_3′ UTR	S (80% paternal X*)
1 Congenital female, 1 Rett-like male	c 378-19delT	Probably none	Intron 3	NI (female*)
1 Angelman like	c 602C→T	p A201V	Interdomain	R
1 Autism with seizures	c 753C→T	p P251P	TRD	S (85% maternal X)
1 Autism	c 1451GC→TC	p R484T	C-terminus	R

FF, forme fruste; MBD, methyl-binding domain; NI, non-informative case; NLS, nuclear localisation signal; PSV, preserved speech variant; R, random X chromosome inactivation; S, skewed X chromosome inactivation; TRD, transcriptional repression domain; UTR, untranslated region; XCI, X chromosome inactivation.
The table includes data on RTT subtype, *MECP2* mutation type and position according to the accepted nomenclature, localisation within the *MECP2* functional domain and pattern of XCI considering preferential activation of the maternal or paternal X chromosome. Mutations were confirmed with the International Rett Syndrome Association database. Cases reported in the lower panel are with inherited *MECP2* polymorphisms or variations with unknown significance.
*Patients were included in quantitative expression assays.

p<0.001 for MECP2_e2); by contrast, the truncating mutations were lower for both isoforms (p<0.001 for MECP2_e1; p = 0.002 for MECP2_e2). The non-truncating mutations were similar to the normal controls for both MECP2 isoforms (p = 1.000 for MECP2_e1; p = 0.161 for MECP2_e2). The overall expression levels of MECP2_e1 and MECP2_e2 were significantly correlated (Spearman r = 0.76; p<0.001).

Quantitative expression analysis of patients negative for *MECP2* mutations

To obtain evidence of *MECP2* mutations in patients with no previous mutation findings, we included 12 such patients in quantitative expression assays, five with classic (C1–C5) and seven with atypical (A1–A7) RTT. We took special care of the transport of blood samples from Canada and the US to ensure that the authentic RNA concentration was maintained (see Methods). Figure 3A,B shows our findings in this group. We correlated the patients with no mutations to the previous groups with mutations, while combining the groups with the splice-site mutations (group I) and the large deletions (group II) on the basis of relative similarity of small groups (p = 0.135 for MECP2_e1 and p = 1.000 for MECP2_e2 by analysis of variance and Bonferroni correction). This analysis suggested that patients C2, C4, C5, A2, A5 and A7 may belong to mutation groups I and II (predicted probabilities to belong to these groups are p = 0.999, 0.999, 1.000, 0.919, 0.993 and 1.000, respectively, including MECP2_e1 and MECP2_e2 in polytomous regression analysis). Patients A3 and A6 were related to group III with the truncating mutations (p = 0.979 and 0.919, respectively). Patient C3 was related to the group with normal expression levels by both MECP2 isoforms (p = 1.000). Patients C1, A1 and A4 had discordant levels of MECP2_e1 and MECP2_e2; in particular one of the isoforms was overexpressed.

Effect of XCI on quantitative expression of MECP2 in peripheral blood

Among the 27 patients included in the quantitative expression assays, seven were detected with considerably skewed XCI. Preferential activation of the paternal X chromosome was detected in patients with the splice-site mutation (donor 1a), the large deletion (del exon 4b), p R270X (XCI data in table 1) and patient C2 (80% XCI). Preferential activation of the maternal X chromosome was found in patients with p

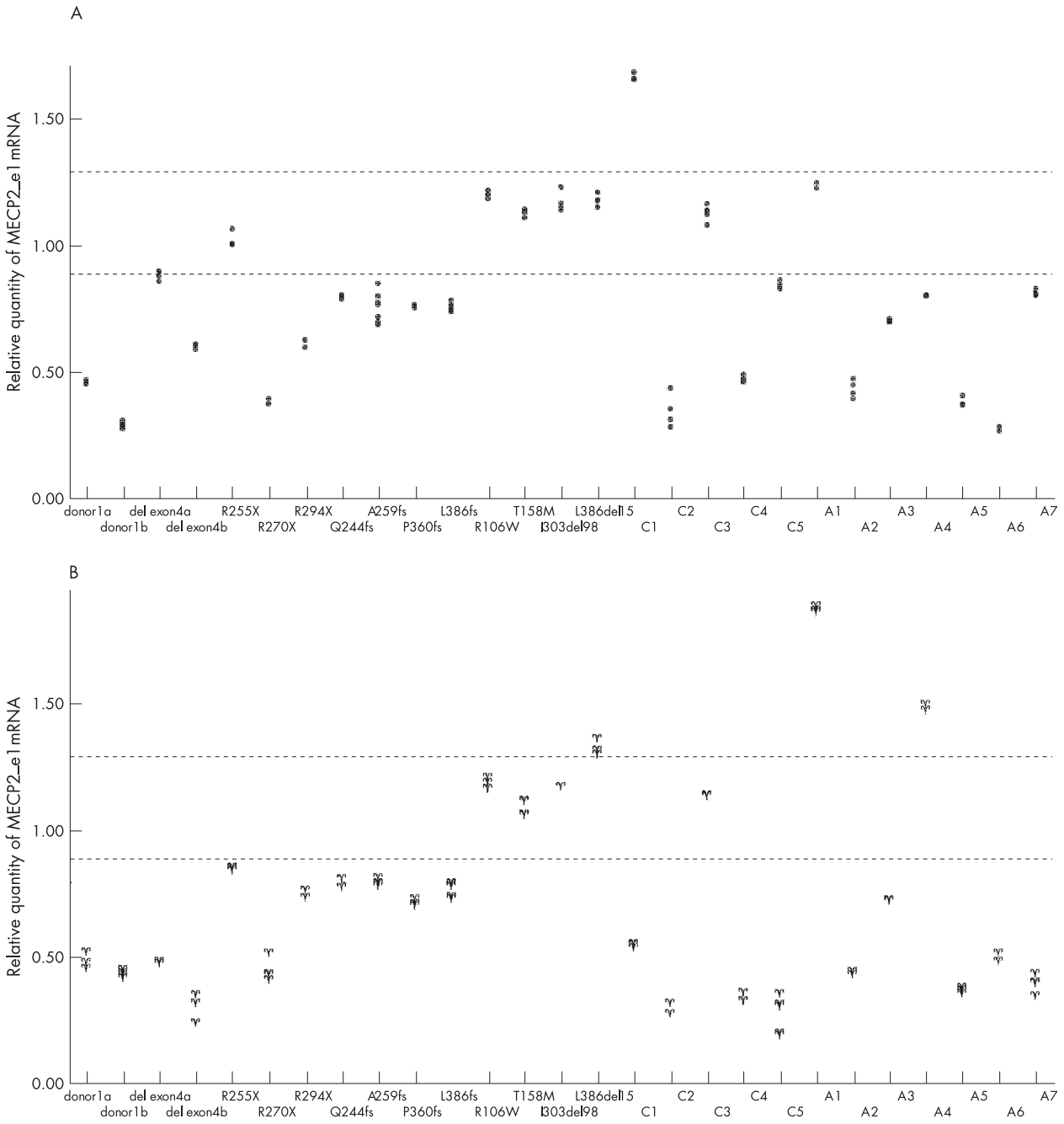

Figure 3 Quantitative analysis of methyl-CpG-binding protein 2 (MeCP2)_e1 and MeCP2_e2 expression levels in blood in patients with Rett syndrome (RTT), using *RNAseP* as the reference gene. The charts include patients with RTT with known *MECP2* mutations and X chromosome inactivation status (table 1), patients with 62+1delGT deletion of intron 1 donor splice site (donors 1a and 1b), deletions spanning exon 4 and the 3′ untranslated region (del exons 4a and 4b), nonsense mutations (R255X, R270X and R294X), frame-shift deletions (Q244fs, A259fs, P360fs, L386fs), mis-sense mutations (R106W, T158M) and in-frame deletions (I303del98, L386del15). In addition, the charts include patients with classic RTT (C1–C5) and atypical RTT (A1–A7) with no *MECP2* mutation findings. Expression levels of (A) MECP2_e1 and (B) MECP2_e2 isoforms were determined according to E^{-ddCt} using *RNAseP* as the reference gene. Dotted lines indicate the normal expression range.

R255X and p R106W (table 1) and patient C4 (80% XCI). Patient C1 was non-informative and other patients had random XCI. We observed interindividual differences between the patients with the same splice-site mutation (donors 1a and 1b), as donor 1b with preferential expression of the paternal X chromosome had lower MECP2_e1 and MECP2_e2 expression levels than donor 1a with random XCI (fig 3A,B). Also, in the two patients with two similar nonsense mutations, p R270X with preferential activation of the paternal X had lower MECP2_e1 and MECP2_e2 expression levels, whereas p R255X with activation of the maternal X chromosome had normal levels of both MECP2 isoforms.

DISCUSSION

This study presents the result of molecular diagnosis of an Israeli cohort of patients with RTT, including various analyses of the *MECP2* gene at the genomic and expression levels. The present cohort included 82 unrelated patients with RTT with classic (n = 52), atypical[17] and related phenotypes.[13] We were

able to provide molecular diagnosis in 80% of classic RTT cases and all PSV variants. The other atypical forms including congenital and forme fruste variants were only partially resolved. No mutations were detected in variants with early seizure onset. Consistent with previous estimates,[7] the recurrent hot-spot mutations comprised about 65% and 3′-end microdeletions comprised an additional 12% of the disease-causing mutations in our cohort. We here report a novel mis-sense mutation c453C→G (p D151E) resulting in a change of a conserved residue at the methyl-binding domain.[18] We also report two maternally inherited polymorphisms, c824T→C (p V275A) and c753C→T (p P251P), the first present in conjunction with a large *MECP2* deletion and the second located at the position where another non-pathogenic mis-sense variation was previously reported.[19] Another rare GT deletion of an intron 1 donor splice site, which has been reported in two other studies,[20 21] was detected in two patients from our cohort.

The question of genotype–phenotype correlation has been answered in a further analysis of patients, aged >5 years, with mis-sense 17 patients and early-truncating mutations (22), whose clinical diagnoses were scored according to the severity scale adopted from Huppke *et al*.[22] Consistent with a previously suggested notion,[23 24] mis-sense mutations were associated with milder RTT phenotypes ($p = 0.002$ by student t-test). Other functional analyses of *MECP2* mis-sense mutations suggest that clinical severity is also dependent on the location of the mutation within the particular *MECP2* functional domain.[25] The contribution of XCI to RTT clinical phenotype is suggested by the finding of a higher proportion of cases with maternally skewed XCI in our cohort, which is similar to observations in other collections and mouse models.[26 27] Although skewed XCI in peripheral blood does not necessarily reflect XCI patterns in the brain, our results may suggest that preferential activation of the maternal X, which in most patients harbours the normal *MECP2*,[28] accounts for milder RTT phenotypes.

The major advance proposed in this study is the examination of expression levels of both MECP2 isoforms in peripheral blood samples from patients with RTT. The question whether quantification of MECP2 expression in vivo might provide yet another molecular indicator of *MECP2* deficiency has been considered by systematic analysis of normal females as well as patients with classic and atypical RTT with known *MECP2* mutations and patients with no mutation findings. Using the quantitative assay that determined the relative expression levels of MECP2_e1 and MECP2_e2 isoforms, we showed that the expression levels of both MECP2 isoforms in normal females fall within a relatively narrow range. Under these conditions, patients with RTT with certain *MECP2* mutations were situated below the normal range (fig 3A,B). We showed distinct effects of various mutations on MECP2 expression levels, and the most marked reduction in the expression levels of both MECP2 isoforms was detected in two patients with ca 62+1delGT deletion of an intron 1 donor splice-site. A previous study showed that this mutation causes a complete skipping of exon 1, resulting in elimination of the MECP2 message and protein in lymphoblast clones of patients with RTT.[29] Large deletions of the *MECP2* 3′-coding region and 3′ UTR also showed lower expression levels, probably as a result of the impairment of 3′-end regulatory sequences important for mRNA processing, polyadenylation and stability.[30] Unlike other mutation types, the effect of large deletions was more evident in the expression levels of MECP2_e2. The functional implications of these findings are not clear and need further replication and analysis. Truncating mutations resulting from early nonsense or frame-shift deletions showed lower MECP2 expression levels that could be attributed to the nonsense-mediated mRNA decay mechanism.[31] The mis-sense mutations and in-frame deletions retained normal MECP2 expression levels, thus supporting the notion that these mutations affect the MECP2 structure and function rather than the level of MECP2 transcripts. Also, the novel p D151E variation had normal MECP2_e1 and MECP2_e2 expression levels using ornithine decarboxylase 1 as an alternative reference gene (data not shown). Another study that examined the in vivo effects of various *MECP2*-coding mutations showed that there were distinctive profiles of histone modifications in peripheral cells in patients with RTT, which may be relevant to neurological dysfunction in RTT.[32]

The interindividual differences in MECP2 expression between carriers of the same or similar mutations could be explained by an additional effect of the XCI pattern. This was particularly evident from the expression levels in two patients with an identical splice-site mutation, in which the patient with a skewed paternal XCI had lower MECP2 (donor 1b). In the same way, maternally skewed XCI minimised the effect of p R255X nonsense mutation and paternally skewed XCI augmented the outcome of p R270X mutation. These findings suggest that peripheral MECP2 expression levels reflect the genetic and epigenetic status of the patient, and thus may be used as yet an additional factor in RTT molecular diagnosis. However, other factors modulating MECP2 mutation expression have been suggested and demonstrated, in particular in males with RTT with *MECP2* truncation mutations.[33] The question whether the peripheral MECP2 expression levels are associated with phenotypic indices and prognosis of RTT should be considered in future studies on a larger series of patients with RTT, with comprehensive characterisation at the clinical and molecular levels.

Ultimately, quantitative expression assays were intended to resolve the diagnosis of patients with RTT with no previous mutation findings. In view of a persistent lack of molecular diagnosis in at least 10% of patients with classic RTT, we proposed that direct estimates of the peripheral MECP2 expression levels could provide alternative indicators of the presence of yet unknown mutations in *MECP2* or other genes that cause MECP2 deficiency. We found that in three of five patients with classic RTT (C2, C4 and C5) and three of seven patients with atypical RTT (A2, A5 and A7), the peripheral MECP2 expression levels were consistent with the presence of splice-site or deletion mutations. Our previous analysis suggested that a lower MECP2_e2 level is specifically associated with the presence of large deletions including *MECP2* 3′ UTR, whereas damage to the splice sites affects both MECP2 isoforms. The question whether this dichotomy is conclusive should be further investigated. We analysed these patients by direct sequencing of candidate regions that are highly conserved and may potentially contain regulatory elements, including 1500 bp surrounding the putative *MECP2* promoter region, and at least 500 bp upstream and downstream of the intronic boundaries, and at least 1500 bp downstream into the 3′ UTR. The major problem with this analysis, however, is the difficulty obtaining samples of both parents to exclude the presence of naive polymorphisms unrelated to RTT. It is also possible that the expected defect is located in other genes that affect MECP2 expression. Our findings of excessive MECP2 expression in several patients (C1, A1 and A4) are ambiguous and need further verification, although recent studies on humans and mice have suggested that over-expression of MECP2 is also pathological.[34 35]

This study essentially stems from the notion that MECP2 deficiency is the central cause of RTT. Although based on a limited number of patients, this study suggests that almost all patients with classic RTT can be related to MECP2 deficiency by systematic analysis of *MECP2* at the genomic or expression level. Yet, even when using such an extended

approach, patient C3 had no indication of MECP2 defect in any of the various tests applied in this study. Today, this patient is 5 years old and is still diagnosed with classic RTT. Although normal MECP2 expression levels do not exclude MECP2-related disease, this case imposes some reservations as to the exclusivity of MECP2 in RTT. The question whether the classic RTT is a single-gene disorder remains open. Nevertheless, our experience suggests that the inclusion of direct MECP2 expression assays in peripheral blood in the molecular diagnostic procedure may provide additional information on genetic and epigenetic conditions associated with MECP2 deficiency and a broader molecular support of RTT diagnosis.

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